Journal of Medical Virology 55:300–304 (1998)

Identification of a Novel Strain of Hepatitis E Virus Responsible for Sporadic Acute Hepatitis in Taiwan

S.Y. Hsieh,[1,2]* P.Y. Yang,[1,2] Y.P. Ho,[1] C.M. Chu,[1] and Y.F. Liaw[1]

[1]Liver Research Unit, Chang Gung Memorial Hospital, Chang Gung University, Taoyuan, Taiwan
[2]Department of Microbiology and Immunology, School of Medicine, Chang Gung University, Taoyuan, Taiwan

Hepatitis caused by the hepatitis E virus (HEV) is a self-limited disease and occurs most frequently as epidemic or sporadic hepatitis in developing countries. The role of HEV in sporadic acute hepatitis in areas without a history of hepatitis E epidemics is obscure. Recently, it was found that more than 10% of the patients with acute non-A, non-B, non-C hepatitis in Taiwan were associated with an acute HEV infection. Nucleotide sequences of the regions within the first open reading frame of HEV were determined in four cases and were 96.7–100% identical to each other. As compared to the isolates from China, Pakistan, Burma, India, Africa, and Mexico, the similarities were, however, only 71.7–79.3%. Phylogenetic analysis revealed that the four Taiwan isolates were categorized as a novel HEV group (the Taiwan strain), which was distinct from all of the strains isolated from other parts of the world. In addition, the isolates from China, Burma, India, and Pakistan were catalogued as the second genotype of HEV (the Asian strain), and the Mexican isolate as the third (the Mexican strain). The African isolate was more related to the Asian type and might be a subtype of the Asian strain. A simple genotyping method by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is described. The findings also support the hypothesis that HEV may be responsible for some sporadic acute non-A, non-B, non-C hepatitis in other developed countries. ***J. Med. Virol. 55:300–304, 1998.***

KEY WORDS: hepatitis E virus; the Taiwan-strain hepatitis E virus; phylogenetic analysis; genotyping

INTRODUCTION

Hepatitis E virus (HEV) has been identified as the major pathogen for enterically transmitted non-A, non-B hepatitis [Reyes et al., 1990; for review see Krawczynski, 1993]. Hepatitis E is endemic and often provokes epidemic outbreaks in developing countries. HEV is also the major cause of the sporadic viral hepatitis in these endemic areas [Krawczynski, 1993]. The role of HEV in sporadic hepatitis in developed countries, however, remains unknown. Only a few cases of sporadic hepatitis E have been reported in developed countries and all had a history of traveling to endemic areas [Bader et al., 1991; Skidmore et al., 1991; Dawson et al., 1992b; Lau et al., 1995]. Hepatitis E is associated with a wide spectrum of liver damage, ranging from mild hepatitis, icteric hepatitis, to fulminant hepatic failure [Krawczynski, 1993]. Host factors, such as pregnancy, age, underlying liver disease, or immune variation are all known to influence the clinical outcome. The viral factors, such as genotypes, still remain to be clarified.

Genome heterogeneity of HCV is known to be the most significant factor affecting the clinical outcome and responsiveness to interferon therapy. Genome heterogeneity of HEV has been previously documented [Yin et al., 1994]. The isolates can be divided into distinct genotypes, which correlate with their geographic distribution. Two major genotypes, the Asian type (the Burmese, Indian, Chinese, and Pakistani isolates) and the North American type (the Mexican isolates) have been described [Aye et al., 1992a, 1992b, 1993; Yin et al., 1994]. The existence of different HEV genotypes has diagnostic and clinical implications, including designing primers for reverse transcriptase-polymerase chain reaction (RT-PCR) for virus detection and for vaccine development. It is also of interest to determine whether there are viral differences between epidemic areas and areas with only sporadic hepatitis E. Taiwan, an endemic area for hepatitis A to D, has had no epidemic episodes of hepatitis E reported. The role of HEV infection in acute sporadic hepatitis in Taiwan is

Contract grant sponsor: the Chang Gung Memorial Hospital; Contract grant number: CMRP726; Contract grant sponsor: the National Science Council, Taiwan; Contract grant number: NSC 862314B182A019MH.

*Correspondence to: Dr. Sen-Yung Hsieh, Liver Research Unit, Chang Gung Memorial Hospital, 199 Tung Hwa North Road, Taipei, Taiwan 105.

Accepted 13 March 1998

unclear. Recently, it was found that more than 10% of our patients with acute non-A, non-B, non-C hepatitis were associated with acute HEV infection [Hsieh et al., unpublished data]. In this article we report the identification of a novel strain of HEV from some patients of sporadic acute hepatitis. The findings suggest the possibility that HEV may also be the pathogen in some sporadic cases of acute hepatitis in other developed countries.

MATERIALS AND METHODS

Patients

Serum samples were collected from four patients with acute hepatitis E.

RNA Extraction and Detection of HEV Viremia by RT-PCR

RNA was extracted from 100 μl of serum by the single-step acid guanidinium thiocyanate-phenol-chloroform method and RNA was converted to cDNA by using a random primer method, as described previously [Hsieh et al., 1997]. Two sets of primer pairs were derived from the first open reading frame of HEV (based on the sequences of the isolates from the Burma, Pakistan, Chinese Xinjiang, and Mexico) for nested PCR. The first-round and the second-round PCRs were done in the same manner for 30 cycles of each. Each cycle entailed denaturing for 40 sec at 94°C, annealing for 1 min at 55°C and extending for 1 min at 72°C. The final products were analyzed by gel electrophoresis and confirmed by Southern blot assay with a digoxigenin-labeled internal oligonucleotide as the probe (Boehringer Mannheim, Germany). The sequences of the outer primer pair were 5′-ATGGAGGCCCATCAGTTTATTA-3′ and 5′-GGCAGTATACCAACGATGAACA-3′; those of the inner primer pair were 5′-GCCGGCCAACTCTGCCCTTGCGAAT-3′ and 5′-CTGGATGGGATGGTTCCAGAAA-3′ ; and those of the internal probe were 5′-GCTGTGGTAGTTAGGCCTTTTCTCTC-3′.

Cloning and Sequencing

The specific PCR products were cloned into the pGEM-T vector in accordance with the commercial instructions (Promega, Madison, WI). Sequences were determined by the dideoxynucleotide method (Sequenase Version 2.0 sequencing kit, United States Biochemical). Three clones were sequenced bidirectionally for each isolate for confirmation of the sequence results.

Phylogenetic Analysis

The nucleotide sequences for the four isolates were compared with those of the Burmese strain (M73218) [Tam et al., 1991], the Pakistani strain (M805821) [Tsarev et al., 1992], the Chinese Xinjiang isolate (L08816) [Aye et al., 1992a, 1992b], the Chinese HeBei isolate (m94177), the Chinese KwangTong isolate (L25595), the Myanma strain (D10330) [Aye et al., 1993], the Africa Moroccan strain (AF010423), and the Mexican strain (M74506) [Huang et al., 1992] using a computer software (Clustal method) with weighted residue weight table (DNAstar, Madison, WI). Percentage of similarity and percentage of divergence were calculated by the method: $M/(M + U + G/2) \times 100$ and $U/(M + U + G/2) \times 100$, respectively, where M is the number of compared positions that are identical, U is the number of positions that are different, and G is the number of positions for which one organism has a base pair and the other does not. A phylogenetic tree was thus constructed on the basis of the data of percentage divergence from the neighboring strains.

RESULTS

The nucleotide sequences of the four Taiwan isolates were aligned with those of the isolates from the three different regions in China, Pakistan, India, Burma/Myanma, Africa Morocco, and Mexico, as shown in Figure 1 and summarized in Table I. The nucleotide sequences of the four Taiwan isolates were 96.7–100% identical to each other, while they were only 71.7–79.3% homologous to any of other strains. The nucleotide sequences of the isolates from the three different areas in China, from India, from Pakistan, and from Burma/Myanma were 98.9–100% identical to each other, while they were 95.7%, 73.9–78.3%, and 80.4% homologous to the Africa Moroccan isolate, any of the four Taiwan isolates, and the Mexican isolate, respectively. The nucleotide sequences of the Mexican strain were 71.7–81.5% identical to any of other strains. The nucleotide sequences of the Africa Moroccan strain were 76.1–79.3%, and 80.4% homologous to any the four Taiwan isolates and to the Mexican strain, respectively. They were, however, 95.7–96.7% similar to the three Chinese, Pakistani, Indian, and Burmese/Myanma isolates.

To further investigate the genetic relatedness of the Taiwan isolates to those isolated from different parts of the world, a phylogenetic analysis was undertaken on the basis of the nucleotide sequences described above (Fig. 2). The isolates from the three different regions (western, northern, and southeastern) of China, along with the isolates from Pakistan, India, and Burma/Myanma, were categorized as a group (the Asian type). The Mexican isolate represented the second group (the North American type). The Moroccan isolate was relatively related to the Asian type, with about a 4% divergence at the nucleotide level, and could be regarded as a subtype of the Asian type. The four Taiwan isolates were categorized as a single, novel group (the Taiwan type) distinct from the other groups mentioned above and from those reported by others.

DISCUSSION

Hepatitis E has been regarded as an infection occurring only as epidemic outbreaks or sporadic cases in underdeveloped countries. In the developed countries, it is generally believed that only those who travel to endemic areas face the risk of HEV infection [Bader et al., 1991; Skidmore et al., 1991; Dawson et al., 1992b;

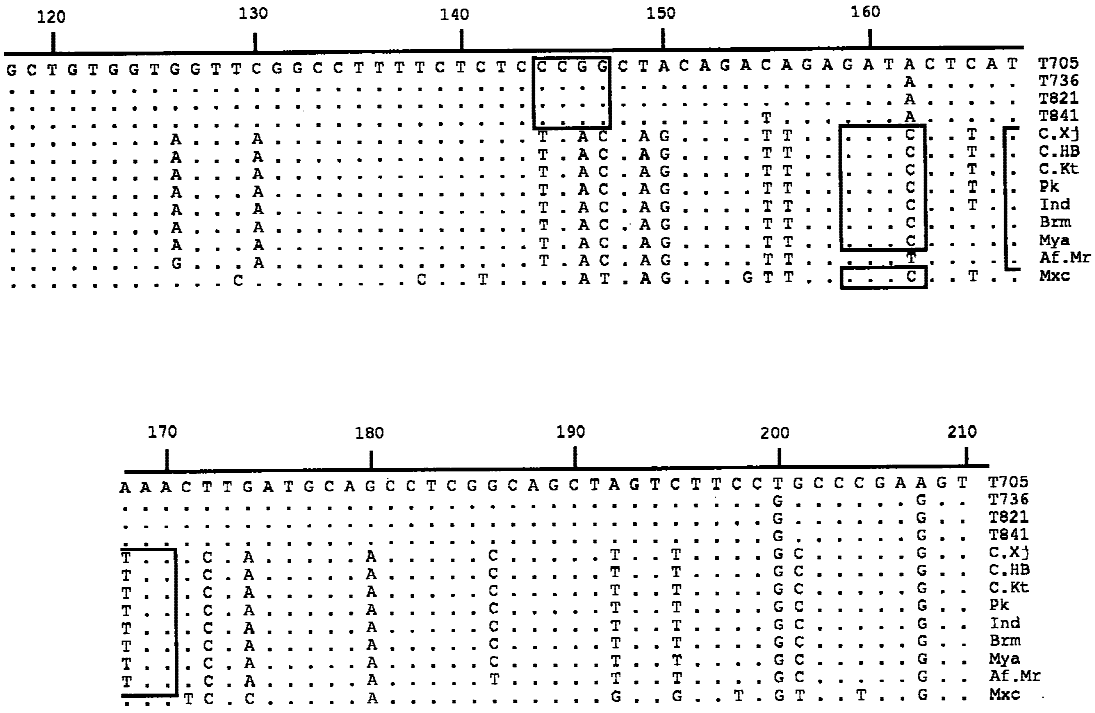

Fig. 1. Alignment of nucleotide sequences of the region within open reading frame 1 of hepatitis E virus genome. T705, T736, T821, and T841 are the four Taiwan isolates; C.Xj, C.HB, C.Kt, Pk, Ind, Brm, Mya, Af.Mr, and Mxc are the isolates from Chinese Xinjiang (L08816), Chinese HeBei (m94177), Chinese KwangTong (L25595), Pakistan (m80582), India (X99441), Burma (M73218), Myanma (D10330), Africa Morocco (AF010423), and Mexico (M74506), respectively. The nucleotide position is in accordance with the numbering system of the Burmese isolate [Tam et al., 1991]. Nucleotide substitutions are in upper-case type, and nucleotides identical to T705 are shown as dots. The nucleotide sequences recognized by restriction enzymes, Hpa II (CCGG; nt 144–147), Sau 3A (GATC; nt 159–162), and Mse I (TTAA; nt 167–170), are indicated by three open squares, respectively.

TABLE I. Identities (%) of the Nucleotide Sequences as Shown in Figure 1

	T705	T736	T821	T841	C.Xj	C.HB	C.Kt	Pk	Ind	Brm	Mya	Af.Mr
T705												
T736	97.8											
T821	97.8	100										
T841	96.7	98.9	98.9									
C.Xj	73.9	76.1	76.1	77.2								
C.HB	73.9	76.1	76.1	77.2	100							
C.Kt	73.9	76.1	76.1	77.2	100	100						
Pk	73.9	76.1	76.1	77.2	100	100	100					
Ind	73.9	76.1	76.1	77.2	100	100	100	100				
Brm	75.0	77.2	77.2	78.3	98.9	98.9	98.9	98.9	98.9			
Mya	75.0	77.2	77.2	78.3	98.9	98.9	98.9	98.9	98.9	100		
Af.Mr	76.1	78.3	78.3	78.3	79.3	95.7	95.7	95.7	95.7	95.7	96.7	
Mxc	71.7	75.0	75.0	75.0	81.5	81.5	81.5	81.5	81.5	81.5	80.4	80.4

Lau et al., 1995]. Nevertheless, anti-HEV was found in a significant proportion, up to 28% in some areas, of healthy individuals in developed countries [Dawson 1992; Thomas et al. 1997]. Whether this was due to subclinical HEV infection or to a nonspecific reaction remains to be clarified. Taiwan, an endemic area for viral hepatitis A to D, has never had a history of epidemic outbreaks of hepatitis E. Recently it was reported that around 10% of the healthy adults in Taiwan were seropositive for IgG anti-HEV, similar to that found in other developed countries [Lee et al., 1994]. Meanwhile, there are still about 10 to 20% of cases of acute hepatitis without a defined etiology. Lee et al. [1994] reported that 46% of patients with acute non-A, non-B, non-C hepatitis in contrast to 10% of patients with acute hepatitis C in Taiwan had serum IgG anti-HEV. The existence of sporadic or subclinical HEV infections in Taiwan was therefore considered. HEV has never been detected, however, in either the serum or feces of any patients with IgG anti-HEV. Recently, it was found that more than 10% of patients with sporadic acute non-A, non-B, non-C hepatitis in Taiwan had HEV viremia and/or IgM anti-HEV [Hsieh et al., unpublished data]. Direct evidence is provided by molecular cloning and sequencing to support the hypothesis that HEV causes some sporadic cases of acute

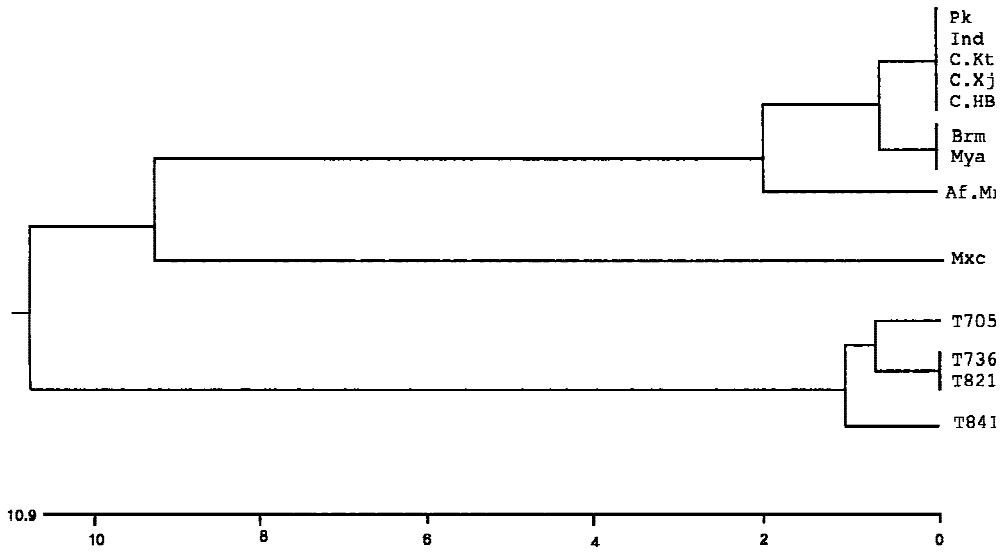

Fig. 2. Phylogenetic analysis of hepatitis E virus isolates from Pakistan (Pk), India (Ind), Chinese KwangTong (C.Kt), Chinese Xinjiang (C.Xj), Chinese HeBei (C.HB), Burma (Brm), Myanma (Mya), Africa Morocco (Af.Mr), Mexico (Mxc), and Taiwan (T705, T736, T821, T841) based on nucleotide sequences within open reading frame 1, as illustrated in Figure 1. Phylogenetic tree was constructed by DNAstar based on the sequence divergence.

hepatitis in Taiwan. This is further supported by the fact that none of the patients had traveled to any hepatitis E endemic area and by the results of phylogenetic analyses.

Phylogenetic analyses revealed three major types and one subtype: the Asian type (including the isolates from western, northern, and southeastern China, and from Pakistan, India, Burma/Myanma), the North American type (the Mexican isolate), the Taiwan type, and the African type (or Asian-African subtype) closely related to the Asian type. Correlation of genetic variations of the HEV to their geographic distribution has also been reported before [Yin et al., 1994]. Two main genotypes—the Asian type (including the Pakistani, the Chinese Xinjiang, the Burmese, and the Myanma isolates) and the North American type of the Mexican isolates—have been proposed [Aye et al., 1992a, 1992b, 1993; Yin et al., 1994]. The Africa Moroccan isolate is considered a subtype of the Asian type and a novel Taiwan type was found. These genotypes can be distinguished by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Within the region of the first open reading frame, as shown in Figure 1 and Table II, the recognition sequences of Hpa III (CCGG) are only present in the Taiwan genotype. The Asian type, the Asia-African subtype, and the North American type can be further differentiated by double digestion with Sau 3A (GATC) and Mse I (TTAA) (cut/cut, uncut/cut, and cut/uncut, respectively).

Nucleotide sequence divergence in the regions, as demonstrated in Figure 1, was noted to be very low among different isolates of the same type. Our findings are consistent with the observations of Aye et al. [1992b] and of Yin et al. [1993] as well. By comparing nucleotide sequences in the 3′ terminal region, Aye et al. [1992b] reported a 92.5% sequence similarities between the Myanma isolates and the Chinese Xinjiang isolates. Yin et al. [1993] found that the nucleotide sequence similarities in nonstructural (RNA-dependent RNA polymerase), as well as structural regions on the HEV genome among the isolates from different parts of Chinese Xinjiang, were around 98–100%. Such a high nucleotide sequence convergence is unexpected in most RNA viruses, since the viral RNA-dependent RNA polymerases do not have proofreading activity to correct nucleotide misincorporation during viral replication. One interpretation is that these are transient infections with HEV without chronicity. A similar phenomenon has also been found with the hepatitis A virus [Cohen, 1989]. The nucleotide divergence between different genotypes of HEV is, however, only about 20–30%. This suggests that HEV has diverged in the world and was introduced into Taiwan a long time ago.

TABLE II. Genotyping by PCR-RFLP[a]

	Hpa II (CCGG)	Sau 3A (GATC)	Mse I (TTAA)
Asian	–	+	+
African	–	–	+
North American	–	+	–
Taiwan	+	–	–

[a]"+", or "–": will be or will not be digested by the enzymes in the region from nt 93 to 235 (in accordance with the numbering system of Tam et al. [1991]).

The mode of transmission of HEV in Taiwan is unknown. Recently, Nanda et al. [1995] reported that HEV viremia could persist up to 4 months after the onset of acute hepatitis in acute sporadic cases in India. It is possible that HEV can survive the interepidemic periods in endemic areas, thereby persistently contaminating water. In the areas without hepatitis E

epidemics, such as Taiwan, however, the transmission routes are even more obscure. While preparing this report, a novel strain of HEV was identified in pigs in the United States by Meng et al. [1997]. A serological survey showed that the majority of swine >3 months of age in herds from the Midwestern United States were positive for anti-HEV. The swine strain HEV was a novel strain distinct from human strains, with 83–92% nucleotide sequences and 77–82% amino acid sequences homologous to human strains. It is intriguing to consider whether the swine HEV can infect humans. Domestic swine were found to be susceptible to experimental infection with a human HEV strain. The nucleotide sequences of the Taiwan strain in the region within open reading frame 1 show a 71.9–79.3% similarity to any other strains of human HEV, consistent with the swine HEV matching. We were unable to clone the region of open reading frame 2 of the Taiwan strain at this stage. As a result, we do not know how close the swine HEV is related to the Taiwan strain HEV, nor the possibility of transmission from swine to cause sporadic cases of acute hepatitis E in Taiwan, an area where pork is the main source of meat protein.

ACKNOWLEDGMENTS

We thank Professor Michael Bullard for critical reading of the manuscript, Miss Ying-Hwa Wu for technical assistance, and Miss Shu-Chen Chi for preparing the manuscript.

REFERENCES

Aye TT, Uchida T, Ma XZ, Iida F, Shikata T, Ichikawa M, Rikihisa T, Win KM (1993): Sequence and genome structure of hepatitis E virus isolated from Myanma. Virus Genes 7:95–100.

Aye TT, Uchida T, Ma XZ, Iida F, Shikata T, Zhuang H, Win KM (1992a): Complete nucleotide sequence of a hepatitis E virus isolated from the Xinjiang epidemic of China. Nucleic Acids Research 20:3512.

Aye TT, Uchida T, Ma XZ, Iida F, Shikata T, Zhuang H, Win KM (1992b): Sequence comparison of the capsid region of hepatitis E viruses isolated from Myanma and China. Microbiology and Immunology 36:615–621.

Bader TF, Krawczynski K, Polish LB, Favorov MO (1991): Hepatitis E in a U.S. traveler to Mexico. Lancet 325:1659.

Cohen JI (1989): Hepatitis A virus: Insight from molecular biology. Hepatology 9:889–895.

Dawson GJ, Chau KH, Cabal CM, Yaubough PO, Reyes GR, Mushahwar IK (1992a): Solid-phase enzyme-linked immunosorbent assay for hepatitis E viurs IgG and IgM antibodies utilizing recombinant antigens and synthetic peptides. Journal of Virological Methods 38:175–186.

Dawson GJ, Mushahwar IK, Chau KH (1992b): Detection of long-lasting antibody to hepatitis E virus in a U.S. traveler to Pakistan. Lancet 340:426–427.

Hsieh SY, Yang PE, Chen HC, Liaw YF (1997): Cloning and characterization of the extreme 5′ terminal sequences of the RNA genomes of GB virus C/hepatitis G virus. Proceedings of the National Academy of Science U.S.A. 94:3206–3210.

Huang CC, Nguyen D, Fernandez J, Yun KY, Fry KE, Bradley DW, Tam AW, Reyes GR (1992): Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology 191:550–558.

Krawczynski K (1993): Hepatitis E. Hepatology 17:932–941.

Lau JY, Sallie R, Fang JW, Yarbough PO, Reyes GR, Portmann BC, Mieli-Vergani G, Williams R (1995): Detection of hepatitis E virus genome and gene products in two patients with fulminant hepatitis E. Journal of Hepatology 22:605–610.

Lee SD, Wand YJ, Lu RH, Chan CY, Lo KJ, Moeckli P (1994): Seroprevalence of antibody to hepatitis E virus among Chinese subjects in Taiwan. Hepatology 19:866–870.

Meng XJ, Purcell R, Halbur PG, Lehman JR, Webb DM, Tsareva TS, Haynes JS, Thacker BJ, Emerson SU (1997): A novel virus in swine is closely related to the human hepatitis E virus. Proceedings of the National Academy of Science U.S.A. 94:9860–9865.

Nanda SK, Ansari IH, Acharya SK, Jameel S, Panda SK (1995): Protracted viremia during acute sporadic hepatitis E viurs infection. Gastroenterology 108:225–230.

Reyes GR, Purdy MA, Kim JP, Luk KC, Young LM, Fry KE, Bradley DW (1990): Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science 247:1335–1339.

Skidmore SJ, Yarbough PO, Gabor KA, Tam AW, Reyes GR (1991): Imported hepatitis E in UK. Lancet 337:1541.

Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE, Reyes GR (1991): Hepatitis E virus (HEV): Molecular cloning and sequencing of the full-length viral genome. Virology 185:120–123.

Thomas DL, Yarbough PO, Vlahov D, Tsarev SA, Nelson KE, Saah AJ, Purcell RH (1997): Seroreactivity to hepatitis E virus in areas where the disease is not endemic. Journal of Clinical Microbiology 35:1244–1247.

Tsarev SA, Emerson SU, Reyes GR, Tsareva TS, Legters LJ, Malik IA, Iqbal M, Purcell RH (1992): Characterization of a prototype strain of hepatitis E virus. Proceedings of the National Academy of Science U.S.A. 89:559–563.

Yin S, Tsarev SA, Purcell RH, Emerson SU (1993): Partial sequence comparison of eight new Chinese strains of hepatitis E virus suggests the genome sequence is relatively stable. Journal of Medical Virology 41:230–241.

Yin S, Purcell RH, Emerson SU (1994): A new Chinese isolate of hepatitis E virus: Comparison with strains recovered from different geographical regions. Virus Genes 92:23–32.